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Human Placental Cytotrophoblasts Produce the Immunosuppressive Cytokine Interleukin 10

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Summary

The mechanism by which the mammalian mother accepts the implanting fetus as an allograft remains unexplained, but is likely to be the result of a combination of factors. Mononuclear cytotrophoblasts, the specialized fetal cells of the placenta that invade the uterus, play an important role. These cells express HLA-G, an unusual major histocompatibility complex class I-B molecule, and secrete cytokines and pregnancy-specific proteins that can regulate immune function. We investigated whether cytotrophoblasts secrete interleukin 10 (IL-10), a cytokine that potently inhibits alloresponses in mixed lymphocyte reactions. Cytotrophoblasts from all stages of pregnancy produced IL-10 in vitro, but neither placental fibroblasts nor choriocarcinoma (malignant trophoblast) cell lines did so. Spontaneous IL-10 production averaged 650, 853, and 992 pg/10^6 cells in the first, second, and third trimesters of pregnancy, respectively. IL-10 secretion dropped ~10-fold after the first 24 h of culture, and was paralleled by a decrease in messenger RNA. IL-10 messenger RNA was detected in biopsies of the placenta and the portion of the uterus that contains invasive cytotrophoblasts, suggesting that this cytokine is also produced in vivo. IL-10 secreted by cytotrophoblasts in vitro is bioactive, as determined by its ability to suppress interferon γ production in an allogeneic mixed lymphocyte reaction. We conclude that human cytotrophoblast IL-10 may be an important factor that contributes to maternal tolerance of the allogeneic fetus.

During human pregnancy, genetically foreign cells from the fetal portion of the placenta invade the uterus. This process is the result of differentiation of the specialized epithelial cells of the placenta, termed trophoblasts (reviewed in 1 and 2). Mononuclear cytotrophoblast stem cells are anchored to basement membranes surrounding the stromal cores of two types of chorionic villi. In floating villi, cytotrophoblasts fuse to form the overlying syncytium, which is in direct contact with maternal blood, mediating nutrient and gas exchange for the developing fetus. In anchoring villi, cytotrophoblasts differentiate by leaving their basement membrane and forming columns of cells. These cell columns give rise to the invasive subpopulation of cytotrophoblasts that attaches to and invades the uterus and its arterial system. In response to cytotrophoblast invasion, the uterine stroma decidualizes and is infiltrated by a specific subset of maternal immune cells. As a result, the pregnant human uterus contains a mixture of fetal and maternal cells, the latter being up to 75% immune-competent bone marrow–derived cells (3).

Because of their unique position at the maternal–fetal interface, placental trophoblast cells are presumed to shield the allogeneic fetus from rejection (4, 5), although the molecular mechanisms have not been fully defined. Faulk et al. (6, 7) suggested that the placenta passively evades immune recognition through the absence of classical class I and II MHC antigens on syncytiotrophoblast cells. However, subsequent studies revealed that invasive cytotrophoblasts express a nonclassical class I antigen, HLA-G (8–10). It has been suggested that this molecule could function as a universal "self"-transplantation antigen, preventing maternal immune attack of the feto-placental unit (9, 11).

The localized secretion of immunomodulatory factors by the placenta may also contribute to survival of the fetal allograft. Hormones in high concentration at the placental–uterine interface, such as progesterone, human placental lactogen, prolactin, and estrogens, inhibit lymphocyte reactivity in vitro (12). Other placental factors with inhibitory effects on immune function include pregnancy-associated α2-glycoprotein, pregnancy-associated plasma protein A,

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and α-fetoprotein (13). In addition to peptide and steroid hormones, the human placenta secretes a variety of cytokines, including CSF-1 (14), M-CSF (15), IL-1β (16), IL-6 (17, 18), and TGF-β (19, 20).

Alloreactive T lymphocyte responses are also suppressed by other, as yet undefined substances in cytotrophoblast-conditioned medium (21) and in the supernatants from cocultures of cyto- and syncytiotrophoblasts (22). This immunosuppressive activity is very similar to the biological effects described for IL-10, an immunoregulatory cytokine that inhibits the generation of alloreactive T cells in mixed lymphocyte reactions (MLR[1]; 23). To test the possibility that IL-10 may be involved in trophoblast-mediated maternal immune inhibition, we investigated the production of IL-10 by several types of human placental cells, including primary cyto- and syncytiotrophoblasts, placental fibroblasts, and choriocarcinoma cell lines. The results of this study show that IL-10 is produced specifically by primary cytotrophoblasts in vitro and that this cytokine can inhibit an allogeneic immune response. We hypothesize that IL-10 may play an important role in suppressing potentially harmful maternal immune responses in vivo.

Materials and Methods

Placental Cell and Tissue Isolation and Culture. Cytotrophoblasts were isolated from pooled first- or second- (24, 25) trimester and individual third-trimester (26) human placentas by published methods. Chorionic villi were collected immediately after vacuum aspiration and washed three times in wash medium (DME H-21, 2.5% FCS, 1% penicillin/streptomycin). Placental fibroblasts were isolated from first-trimester placentas as previously described (24). The JAR (27), BeWo (28), and JEG (29) human choriocarcinoma cell lines (American Type Culture Collection, Rockville, MD) were maintained as previously described (24).

Cytotrophoblasts, choriocarcinoma cell lines, and fibroblasts (10^6) and chorionic villi (0.1 g) were cultured in 1 ml of serum-free medium (DME H21 MEM containing 2% Nutridoma [Boehringer Mannheim, Indianapolis, IN] and 50 μg/ml gentimicin; serum-free medium) in 16-mm culture wells precoated with a basement membrane substrate (Matrigel; Collaborative Research Inc., Bedford, MA) diluted two parts to one part SFM as described (25). For immunohistochemical studies, cells were plated at a density of 2.5×10^5 cells/ 500 μl SFM in each well of an eight-well chamber slide (Nunc, Inc., Naperville, IL). PBMC were cultured in RPMI 1640 supplemented with 10% heat-inactivated human AB serum.

Where specified, IL-10 production was stimulated by the addition of 1 μg/ml LPS from *Escherichia coli* (Serotype No. 0127:B8; Sigma Immunochemicals, St. Louis, MO) to SFM. For metabolic labeling, 10^6 cytotrophoblasts were cultured for 12 h in 1 ml cysteine- and methionine-deficient DME containing 500 μCi Tran^{35}S-label™ (ICN Biomedicals Inc., Costa Mesa, CA).

Quantification of IL-10 in Conditioned Medium (CM) and Cell Extracts. CM was collected after 24 h and centrifuged at 300 *g* for 8 min. CM was then concentrated using Centriprep (5–15 ml medium) or Centricon (up to 3 ml medium) concentrators with a 10,000–mol wt cutoff (Amicon, Inc., Beverly, MA). Cell extracts were prepared by adding 1 ml lysis buffer (0.5% NP-40, 150 mM NaCl, 25 mM Tris-HCl, pH 7.5) containing 1 mM PMSF to the cultures. The resulting suspensions were passed 10 times through a 26-gauge needle. Lysates were then centrifuged at 16,000 *g* for 5 min to remove cellular debris and filtered through a 0.2-μm filter. CM and lysates were stored at −80°C. IL-10 levels were determined by an ELISA developed in this laboratory as previously described (30–32). The sensitivity of the assay is 50 pg/ml.

Immunocytochemistry. Cells cultured overnight in chamber slides were fixed for 15 min in acetone at −20°C, then incubated in methanol containing 0.3% H_2O_2 for 10 min to block endogenous peroxidase activity. After a 1-h incubation in antibody diluent (PBS containing 0.7% gelatin from cold-water fish skin; Sigma), a mixture of 25 μg/ml each of two mAbs to human IL-10 (19F1 [33] and 12G8 [31, 33]) was added for 48 h at 4°C. We were unable to detect IL-7 in cytotrophoblast conditioned medium by ELISA (data not shown). Consequently, as a negative control, cytotrophoblasts were exposed to 50 μg/ml of a rat mAb to human IL-7 (BVD10-11C10; 33) that was of the same IgG subclass as both 19F1 and 12G8. As an additional control, cells were exposed to antibody diluent alone. Slides were incubated with a 1:100 dilution of biotinylated goat anti–rat IgG (Vector Laboratories, Inc., Burlingame, CA) for 60 min at room temperature. ABC reagent solution (Vector) was then added for 60 min, and the peroxidase reaction was developed by incubating with 3,3′-diaminobenzidine (Vector) containing NiCl. Stained cytotrophoblasts were examined by bright-field optics with an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) and photographed with Kodachrome 160 ASA tungsten film (Eastman Kodak Co., Rochester, NY).

Immunoprecipitation. 10^6 metabolically labeled cells were extracted in 1 ml 2.5% Triton dilution buffer (TDB; 34) containing 5 μg/ml pepstatin, 10 μg/ml chymostatin, 5 μg/ml leupeptin, 10 μg/ml antipain, 0.5 nM benzamidin, 0.5 U/ml trasylol, and 1 mM PMSF. CM (1 ml) was added to 0.5 ml TDB, and both medium and labeled cells were frozen at −80°C before use (1–7 d). Lysates and conditioned media were precleared twice with CL2-B beads (Pharmacia LKB Biotechnology, Piscataway, NJ) as described (35). As an additional preclearing step, supernatants were added to 50 μl of a 30% (vol/vol) slurry of protein A-Sepharose (PAS) beads in PBS containing 0.03% NaN_3 and incubated with a rat IgG_2a mAb to β-galactosidase (GL117, 10 μg/ml) for 6 h at 4°C with continuous agitation. After 30 min, rabbit anti–rat IgG (10 μg/ml; Vector) was added to each tube. The precleared samples were then incubated with PAS and 3.3 μg/ml each of three mAbs to human IL-10 (12G8, 9D7 [31], and 19F1 [33]) with constant agitation for 12 h at 4°C. Rabbit anti–rat IgG (10 μg/ml; Vector) was added after 30 min. PAS beads were then pelleted by centrifugation and washed five times with mixed micelle buffer and three times with final wash buffer (FWB; 35), with a transfer of the beads to clean tubes during the second FWB wash. Beads were boiled for 3 min in Laemmli sample buffer containing 2.5% β-mercaptoethanol and subjected to SDS-PAGE on a 15% acrylamide gel. The gel was fixed for 10 min in glacial acetic acid, incubated for 1 h in EN^{3}HANCE (DuPont Co., Wilmington, DE), rinsed twice in tap water, and incubated in H_2O containing 3% glycerol for 1 h to precipitate the fluor. The gel was then dried and exposed to X-OMAT AR film (Kodak).

Analysis of Cytotrophoblast IL-10 mRNA. mRNA was extracted from 10^6 cells using RNAzol™ B (Biotecx Laboratories, Inc., Houston, TX) according to the manufacturer's recommenda-

[1] *Abbreviations used in this paper:* CM, conditioned medium; HPRT, hypoxanthine-guanine phosphoribosyltransferase; MLR, mixed lymphocyte reaction.

tions. RNA was reverse transcribed with murine Moloney leukemia virus reverse transcriptase (GIBCO BRL, Bethesda, MD) and random hexamer primers (Sigma) as previously described (36, 37). To quantitate levels of IL-10 mRNA, cDNA was amplified in the presence of a multiple human cytokine competitor plasmid otherwise identical to a murine construct described in detail elsewhere (36, 37). Briefly, the competitor plasmid, called DC10, consists of tandemly arrayed authentic target sequences into which DNA spacers of 75–150 bp were introduced. Target sequences in the competitor construct amplify with equivalent efficiency in the presence of cDNA, and are easily distinguished from the smaller wild-type amplimers by 2% agarose gel electrophoresis and ethidium bromide staining. A competitor for the constitutively expressed gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), was included in DC10, allowing input cDNA to be standardized to comparable levels of HPRT expression in each sample.

The first step in quantitating IL-10 mRNA was to determine the amount of HPRT cDNA in each sample as a relative measure of total cDNA. In these initial PCR reactions, equal volumes of DC10 were added to equal volumes of each of the cDNA samples. The reaction products were separated by electrophoresis and visualized by ethidium bromide staining. Samples containing large amounts of cDNA relative to DC10 resulted in a brighter wild-type band. Next, total cDNA levels were equalized among samples by adjusting the amounts of input cDNA while maintaining a constant concentration of DC10. In samples with a relatively high concentration of cDNA, a smaller volume was used in successive reactions until the ratio of band intensities between competitor and wild-type HPRT amplimers was identical in each sample. To determine the relative levels of IL-10 transcript in each sample, equal amounts of total cDNA, whose volumes were determined previously, were then used for amplification of IL-10. Competition for reagents between the competitor, whose concentration remained constant, and varying amounts of IL-10 cDNA in each reaction resulted in different ratios of band intensities. As cDNA levels decreased, the larger competitor construct was preferentially amplified, resulting in a brighter band. These relative amounts of wild-type and competitor reaction products provide a semiquantitative determination of relative levels of IL-10 mRNA in each sample. Primer sequences were as follows: HPRT 3′: CCTGCTGGATTACATCAAAGCACTG; HPRT 5′: TCCAACACTTCGTGGGGTCCT; IL-10 3′: ATGCCCCAAGCTGAGAACCAAGACCCAGAC; IL-10 5′: TCTCAAGGGGCTGGGTCAGCTATCCCA.

PCR of Placental Biopsies. Total RNA was isolated from frozen, pulverized biopsies of term placenta and placental bed by using RNAzol™ B (Biotecx Laboratories, Inc., Houston, TX) according to the manufacturer's recommendations and reverse transcribed as described above. IL-10 cDNA was amplified using primers shown above.

MLR. PBMC were prepared by centrifugation of blood from normal donors on a Ficoll-Hypaque 1077 (Sigma) density gradient according to the manufacturer's recommendation. 10^5 PBMC (responders) were mixed with 10^5 irradiated (3,000 rad) allogeneic PBMC (stimulators) and cultured in triplicate in round-bottom microtiter plates (200 μl/well). In some wells, CM from individual preparations of cytotrophoblast cells was added at a final concentration of 50% (vol/vol). Where specified, CM was preabsorbed with 20–0.15 μg/ml of a function-perturbing antibody to IL-10 (anti–IL-10, 19F1; 23, 38) or an anti–β-galactosidase antibody matched for IgG subclass (control IgG, GL117; 31) for 3 h at 37°C before addition. On day 5, the medium was harvested and centrifuged for 10 min at 300 *g*. An ELISA specific for IFN-γ, developed in this laboratory (32), was performed to determine levels of this cytokine in the culture medium. The sensitivity of the assay is 50 pg/ml. Data were analyzed using the Mann-Whitney *U* test for nonparametric distribution.

Results

Human Placental Cytotrophoblasts Secrete IL-10 In Vitro. We first examined cytokine synthesis by a variety of placental cells, using ELISA. To test cytotrophoblast production of cytokines, we used an in vitro system shown by stage-specific antigen expression (reviewed in 1 and 2) to model the differentiation of these cells along the invasive pathway in vivo. To obtain the required number of cells, first-trimester cytotrophoblasts were isolated from 5–10 pooled placentas (n = 16), second-trimester cells from 1–4 pooled placentas (n = 14), and term cells from a single placenta (n = 8). Cytotrophoblast populations prepared in this way are 95% free of CD-45–positive immune cells, themselves potential cytokine-producing contaminants (25). We also analyzed CM from organ cultures of intact chorionic villi. Under these conditions, syncytiotrophoblasts are the primary source of secreted molecules. Cytokine production by other placental cell types, including first-trimester placental fibroblasts and the BeWo, JAR, and JEG choriocarcinoma cells lines, was also investigated.

We recently showed that purified cytotrophoblasts produce IL-1β, with highest levels secreted during the first trimester of pregnancy (16). In the present experiments, we confirmed previous reports that these cells produce IL-6, but neither IL-2, -3, -4, -5, -7, nor IFN-γ were detected by ELISA in CM (data not shown). In contrast, all cultures contained IL-10 in levels that were >50 pg/ml, the minimum sensitivity of the assay (Fig. 1). There were no signif-

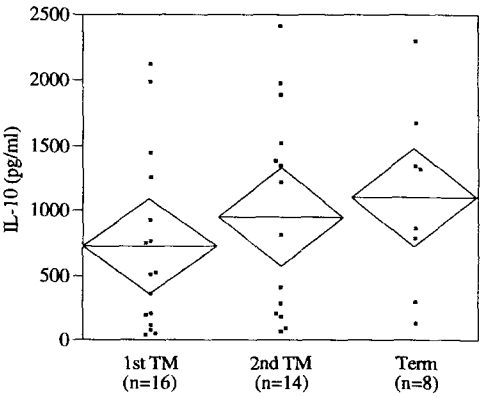

Figure 1. IL-10 levels secreted by cultured human cytotrophoblasts. Cytotrophoblasts were isolated from pooled first or second trimester (*TM*) or individual term human placentas. 10^6 cells were cultured in 1 ml serum-free medium. After 24 h, CM was collected, and levels of secreted IL-10 were quantified by ELISA. Each point represents the level of IL-10 in the CM of one preparation of cytotrophoblast cells. Diamonds signify means with 95% confidence intervals.

icant differences in the mean IL-10 levels produced by cytotrophoblasts from different gestational ages. However, there were substantial differences in the absolute amount of IL-10 secreted by cells obtained from different pregnancies. Human immune cells secrete IL-10 in vitro after activation with agents such as endotoxin (30). Although cytotrophoblasts secreted IL-10 without stimulation, addition of 1 μg/ml LPS to first-trimester cells increased IL-10 secretion approximately threefold compared to untreated cells from the same preparation. With regard to other placental cell types, 0.1 g of chorionic villi produced ~2 ng IL-6, but less than 50 pg of IL-10, suggesting that syncytiotrophoblasts do not produce considerable quantities of the latter cytokine. In addition, neither the CM nor lysates of placental fibroblasts or choriocarcinoma cells contained IL-10. These data suggest that cytotrophoblasts are the major placental source of IL-10 in vitro and that the choriocarcinoma cell lines do not secrete this cytokine.

We used a second experimental approach to verify that human cytotrophoblasts synthesize IL-10. Purified cells were cultured for 12 h and immunostained with mAbs specific for IL-10. An mAb to IL-7 that was matched for IgG subtype was used as a control. Purified cytotrophoblasts form aggregates during the first 48 h of culture when plated at subconfluent levels (25). A subset of such cells reacted with IL-10 antibodies (Fig. 2 *A*), but not with IL-7 anti-

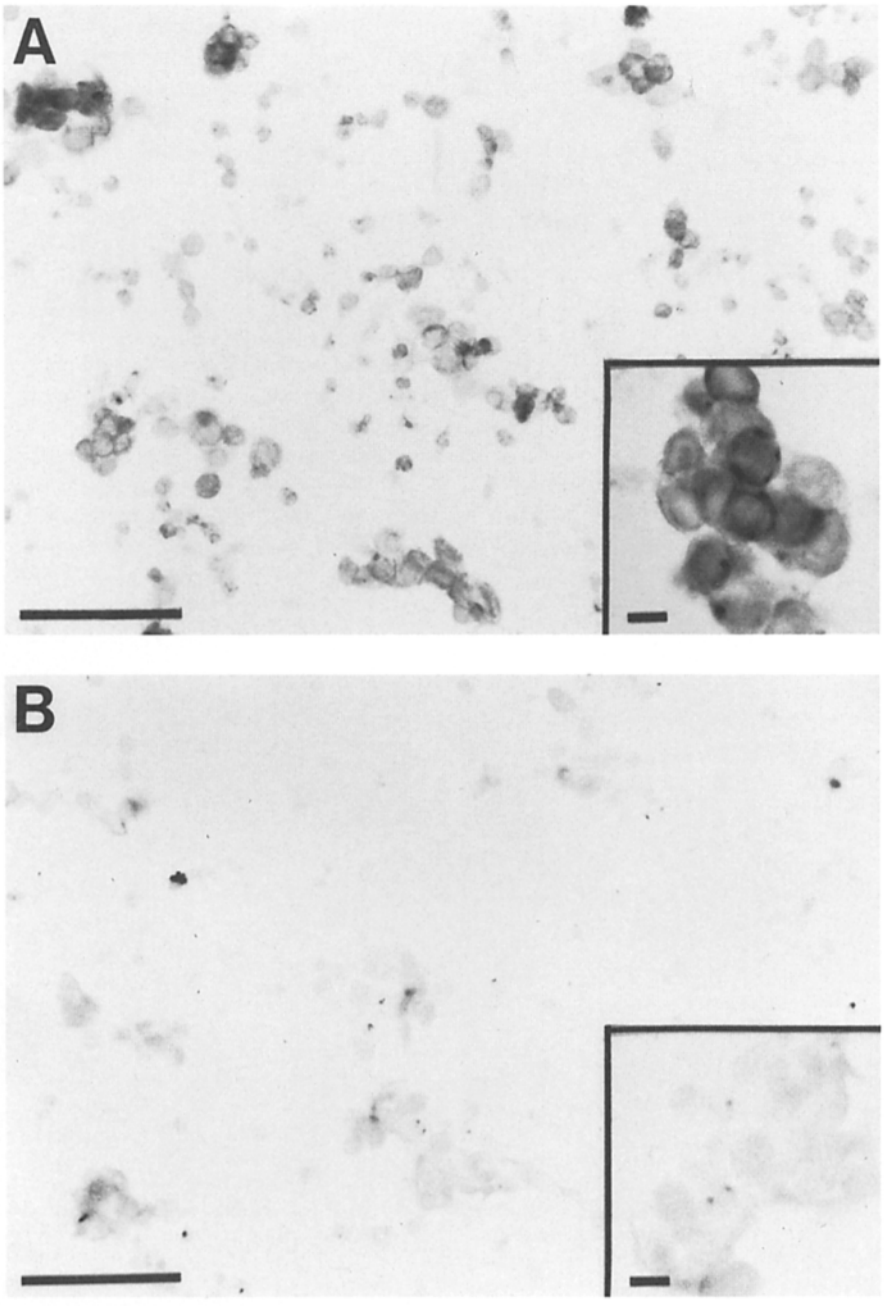

Figure 2. Immunodetection of IL-10 in cultured human cytotrophoblasts. 2.5×10^5 first-trimester cytotrophoblasts were cultured in 500 μl serum-free medium. After 12 h, cells were fixed and stained with mAbs to IL-10 (*A*) or IL-7 (*B*), as described in Materials and Methods. Bar, 100 μm; inset bar, 10 μm.

bodies (Fig. 2 *B*). In accordance with the ELISA data, placental fibroblasts and the choriocarcinoma cell lines did not stain positively for IL-10.

Temporal Regulation of IL-10 Production. To establish that cytotrophoblasts produce, as well as secrete, IL-10 in vitro, we immunoprecipitated this protein from lysates and CM prepared from metabolically radiolabeled cells (Fig. 3). Autoradiography of the electrophoretically separated immunoprecipitates showed a single band corresponding to the molecular mass of human IL-10 (18 kD). This band was most prominent in CM collected from cells that had been cultured for 12 h, and it decreased in intensity over the 60-h culture period. Cell lysates contained much lower, but still detectable levels of IL-10, and again the highest amount was seen after 12 h in culture. No specific bands were detected in immunoprecipitates obtained using an antibody to IL-7 (25 kD). These results suggest that cytotrophoblast synthesis of IL-10 is highest during the first 12 h, and that this cytokine is rapidly secreted into the medium. This was verified by ELISA, which showed that the amount of IL-10 secreted by cytotrophoblasts dropped after 24 h to levels that were below the sensitivity of the assay. During this same period, cytotrophoblast cells cultured under identical conditions remain viable as indicated by their continued secretion of placental hormones (e.g., human chorionic gonadotropin; 24) and by their expression of newly synthesized stage-specific antigens (reviewed in 1 and 2).

Mechanism of Downregulation of IL-10 Synthesis. To elucidate the mechanism by which protein synthesis is downregulated, we used a competitive PCR-based method with primers specific for human IL-10 to measure mRNA levels in cultured cells. We were unable to detect message for this cytokine in samples from control cultures of placental fibroblasts or the BeWo, JAR, and JEG cell lines. IL-10 message was, however, readily detected in first-, second-, and third-trimester cytotrophoblasts (Fig. 4) both at the time of isolation (time 0) and after as long as 2 d in vitro (48 h). In accordance with the immunoprecipitation and ELISA data, the amount of IL-10 mRNA (as normalized to HPRT) decreased with time in culture. Together, these data suggest that the decline in IL-10 production in vitro is transcriptionally regulated.

The presence of IL-10 mRNA in cytotrophoblasts immediately after purification suggests that these cells either synthesize IL-10 in vivo or are induced to do so by the isolation procedure. To begin to address this question, we used RT-PCR to analyze various tissues that are components of the maternal-fetal interface. Both chorionic villi and the portion of the uterus to which anchoring villi attach expressed IL-10 mRNA (data not shown). Since these specimens contain a variety of cell types in addition to cytotrophoblasts, it remains to be determined which of these cells produce IL-10 in vivo.

Cytotrophoblast IL-10 is Immunoinhibitory. Next, we investigated whether it was possible to detect one biological activity of IL-10, namely the ability to inhibit an allogeneic immune response, within the complex mixture of growth factors and cytokines that cytotrophoblasts secrete. Specifically, we measured the ability of cytotrophoblast CM to inhibit IFN-γ production in an MLR. In control experiments, the addition of 1-100 U rIL-10 to an MLR inhibited IFN-γ secretion in a dose-dependent manner. As previously shown, as little as 1 U rIL-10 was sufficient to reduce IFN-γ

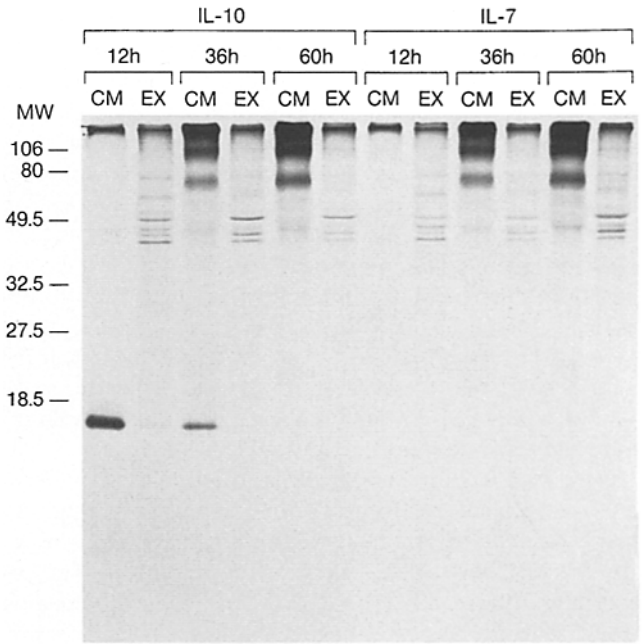

Figure 3. Human cytotrophoblasts synthesize IL-10 in vitro. Term cytotrophoblasts were metabolically labeled for 12 h beginning at 0, 24, and 48 h in culture. CM was collected, and a cell extract (*EX*) was prepared. Samples were incubated overnight with mAbs to IL-10 or, as a negative control, an mAb to IL-7. The resulting immune complexes were separated on a 15% polyacrylamide gel and exposed to film for 3 d.

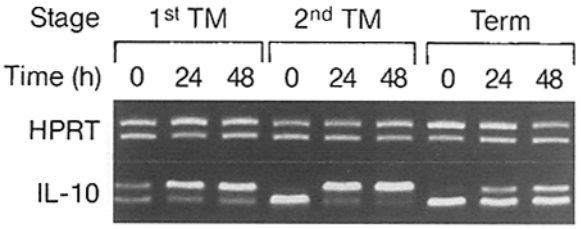

Figure 4. Relative levels of IL-10 mRNA in human placental cytotrophoblasts over time in culture. Placental cytotrophoblasts were isolated as described in Materials and Methods from pooled first or second trimester (*TM*) or individual term placentas, and RNA was extracted and reverse transcribed. cDNA was amplified by PCR in the presence of the competitor plasmid, DC10 (see Materials and Methods), allowing the relative levels of IL-10 mRNA to be quantified by comparing the relative amounts of the larger competitor sequence (*upper band in each lane*) and the wild-type transcript (*lower band in each lane*). The equal relative intensities of cDNA and competitor HPRT amplimers in each sample (*upper panel*) indicate that an equal amount of input cDNA was added to each reaction. IL-10 transcripts were then quantitated by using these equalized amounts of input cDNA in PCR reactions containing a constant amount of DC10 (*lower panel*). Over the course of this experiment (48 h), the larger competitor construct became preferentially amplified, indicating that IL-10 mRNA levels decreased in cytotrophoblasts of all gestational ages with time in culture. Results are representative of two experiments.

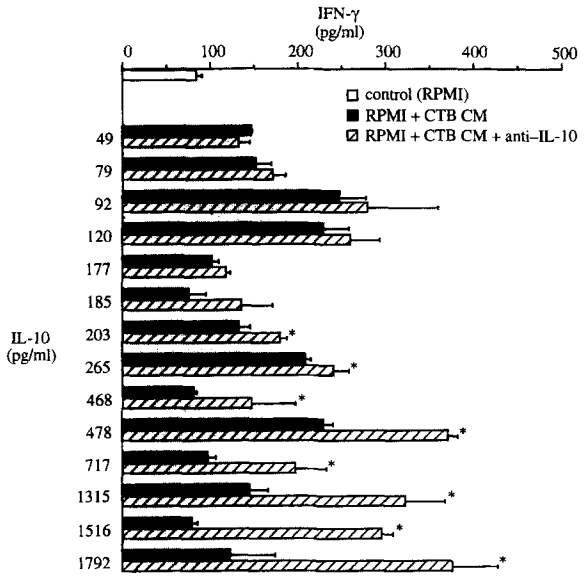

Figure 5. IL-10 in cytotrophoblast CM inhibits IFN-γ secretion in a primary MLR. Cytotrophoblast CM (*CTB CM*) was collected after 12 h from individual preparations of cells, and absolute amounts of IL-10 in each sample were determined by ELISA. Alloreactive lymphocytes were cultured in triplicate as described in Materials and Methods in the presence of RPMI alone (*white bar*) or 50% (vol/vol) CTB CM from individual cell preparations either untreated (*black bars*) or preabsorbed with 10 μg/ml of a neutralizing mAb to IL-10 (*hatched bars*). After 5 d, CM was collected and IFN-γ levels in triplicate cultures were determined by ELISA. Data of all experiments are presented as mean ± SEM. Asterisks denote significant increases in IFN-γ secretion ($P < 0.05$ by the Mann-Whitney *U* test for nonparametric distribution).

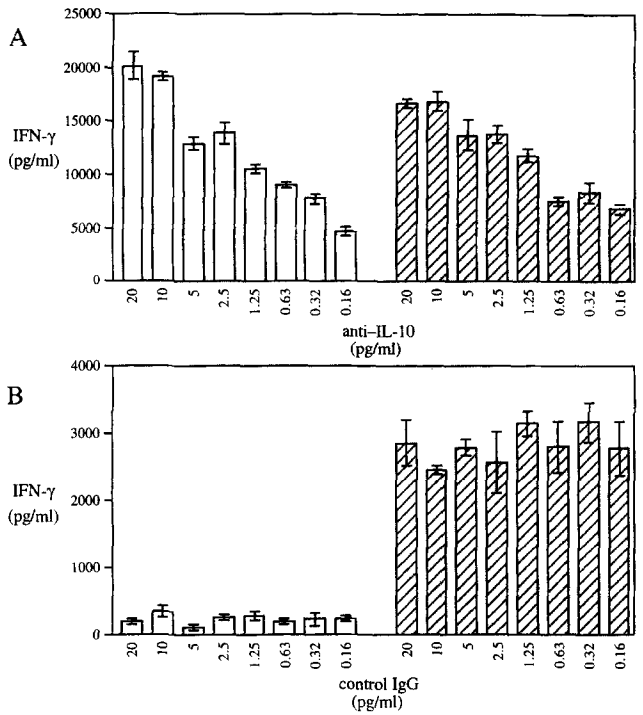

Figure 6. Dose–response inhibition of IFN-γ secretion by cytotrophoblast IL-10. CM was collected from cytotrophoblast cells after 12 h in culture. Alloreactive lymphocytes were cultured in triplicate as described in Materials and Methods in the presence of 50% (vol/vol) CM or 1,000 pg/ml rIL-10, each preabsorbed with 20 to 0.15 μg/ml of a neutralizing mAb to IL-10 (anti–IL-10; *A*) or an isotype-matched control antibody (control IgG; *B*). Results shown are from two individual experiments and represent mean ± SEM. □, 1,000 pg/ml rIL-10; ▨, CTB CM.

levels by ~25%, and the function-perturbing antibody 19F1 reversed this suppressive activity (23). In addition, 19F1 increased basal IFN-γ levels by neutralizing endogenous IL-10 when added alone to an MLR. These effects were not observed when a nonspecific antibody of the same IgG subtype was used as a control. Addition of CM from different preparations of cytotrophoblasts to an MLR had variable effects on IFN-γ production (Fig. 5). However, neutralizing IL-10 in cytotrophoblast CM resulted in a significant increase in IFN-γ secretion in many samples ($n = 8$, $P < 0.05$). In these experiments, the ability to stimulate IFN-γ production positively correlated with the amount of IL-10 in the CM (correlation coefficient $R^2 = 0.86$). Titrating 19F1 in the CM of one preparation of cytotrophoblasts resulted in a dose-response stimulation of IFN-γ production (Fig. 6 *A*). Again, the isotype-matched control antibody had no effect (Fig. 6 *B*). These observations show that IL-10 secreted by cytotrophoblasts acts to inhibit allogeneic lymphocyte reactivity in vitro.

Discussion

We report that IL-10 secreted by highly purified human placental cytotrophoblasts can suppress an allogeneic immune response in vitro. Human IL-10 is a pleiotropic cytokine that is produced by activated immune cells including human $CD4^+$ T cell clones (Th0, Th1, and Th2 cells; 39), B cells (40), and monocytes (30), by UV-irradiated murine keratinocytes (41), and by a variety of human carcinoma cell lines (38). Among its activities, IL-10 suppresses both T cell proliferative responses and IFN-γ production in MLRs when allogeneic cells are used as stimulators (23). IL-10 suppresses alloresponses by inhibiting the expression of both MHC class II molecules and the costimulatory molecule B7 (42, 43). Alloreactive T cells, such as those generated in these reactions, play a key role in allograft rejection (44). These effects of IL-10 make it an attractive candidate for suppressing transplant rejection and prolonging graft survival (45).

To investigate the possibility that IL-10 may be involved in protecting the allogeneic fetal "transplant" in human pregnancy, we determined whether placental cells synthesize this cytokine. Of the cells we studied, only primary cytotrophoblasts secreted IL-10 in vitro, in levels ranging from 67 to 2,190 pg/ml during the first 24 h of culture. Our findings correlate with IL-10 production in several human diseases, in which systemic cytokine levels are equally variable. For example, serum levels of IL-10 in patients with meningococcal septic shock (46) or diffuse large cell lymphoma (47) range from 25 to 64,500 pg/ml and ≤5

to 27,143 pg/ml, respectively. Even an inbred population of mice injected with an antibody to CD3 displays a ninefold variation in serum concentrations of IL-10 (48). The intersample variability of IL-10 production by cytotrophoblasts is also reflective of the biologic variation in pregnancy-specific hormone production by different placentas. For example, the amount of human placental lactogen (hPL) released by cultured placentas covers a fivefold range of values (49). This is also observed in vivo, where circulating levels of hPL in normal pregnancy range from 3.3 to 25 μg/ml in the third trimester (50). As each placenta is the product of a unique immune and hormonal environment, it follows that the production of IL-10 will be regulated differently in each pregnancy.

Purified cytotrophoblasts contain IL-10 mRNA immediately after the isolation procedure, before culture. Both IL-10 gene transcription and subsequent protein secretion drop within 24 h in vitro. During this time, cytotrophoblasts are actively differentiating as they do during uterine invasion in vivo; they modulate the expression of molecules whose functions mediate either maternal immune tolerance (e.g., HLA-G; 10) or invasion (e.g., integrin cell-ECM receptors [51] and MMP-9 [24]). We have evidence that IL-10 can affect both of these important functions. With regard to immune aspects of pregnancy, we show here that IL-10 in cytotrophoblast CM inhibits alloreactivity. In addition, preliminary data suggest that endogenous IL-10 downregulates the production of MMP-9, which is required for invasion in vitro (25).

It has been suggested that the maternal immune system in pregnancy is biased towards antibody production, while harmful cell-mediated immunity is weakened (52). The generation of a humoral response is associated with the production of Th2-type cytokines, including IL-3, IL-4, IL-5, IL-10, and IL-13. Of these, IL-10 directly inhibits synthesis of proinflammatory Th1-type cytokines (e.g., IFN-γ), thus preventing the development of cytotoxic immune cells (39, 53). Several groups have demonstrated the production of Th2 cytokines by tissues at the maternal–fetal interface in murine gestation (54, 55). However, the precise identity of the cell types producing these cytokines has not been elucidated. This report shows that in human pregnancy, cytotrophoblasts secrete IL-10. Consistent with the ability of IL-10 to inhibit the production of Th1 cytokines, we did not detect IFN-γ in purified cytotrophoblasts by any of several methods used (56). We postulate that the localized production of IL-10 by the placenta serves to protect the human fetus by driving the maternal immune system away from a potentially deleterious cell-mediated Th1 response.

For IL-10 to function as a local immunoinhibitor in pregnancy, its levels within the uterine wall must be sufficient to suppress a harmful maternal immune response. Since 100 pg/ml rIL-10 is sufficient to inhibit T cell proliferation in an MLR (23), the amount of IL-10 secreted by these cells in vitro is in a physiologically relevant range. In addition, we and others have detected IL-10 mRNA in biopsies of both placenta (57) and placental bed, the decidualized uterine tissue into which cytotrophoblasts invade. IL-10 has also been demonstrated to be present in human amniotic fluid (58). Together, these observations suggest that this cytokine could be produced in sufficient amounts to be active in vivo. We have also shown that cytotrophoblast-derived IL-10 is immunosuppressive in vitro, as determined by its effect on IFN-γ secretion in an MLR. The fact that we detected this activity in the complex growth factor and cytokine mixture in CM suggests that IL-10 could play an important role in maintaining the fetal allograft.

The significance of placental IL-10 production in vivo is difficult to assess in animal models because many aspects of human pregnancy are unique. For example, human mononuclear cytotrophoblasts behave like tumor cells in that they migrate deep into the uterine wall, mixing extensively with maternal decidual cells (2). Mouse placentation is characterized by shallow invasion by multinucleate giant cells, which results in the formation of a distinct fetal–maternal boundary (2). Human pregnancy is also immunologically unique, as evidenced by the fact that invasive cytotrophoblasts express HLA-G (8–10). It is not known whether there is a mouse HLA-G homologue. In addition, the substantial difference in the length of gestation in the two species (20 d in the mouse vs. 40 wk in the human) suggests that different immunomodulatory mechanisms might be required for protection of the allogeneic fetus. Therefore, the successful birth of IL-10–deficient mice (59) does not exclude the possibility that this cytokine is important for the maintenance of human pregnancy. In fact, recent evidence suggests that IL-10 production is disregulated in some reproductive pathologies. IL-10 levels are significantly elevated in the amniotic fluid of small-for-gestational-age pregnancies, these being the leading cause of perinatal morbidity and mortality (58). Additionally, PBMCs isolated from women suffering from recurrent spontaneous abortions were found to secrete the Th1 cytokines IFN-γ, IL-2, TNF-β, and TNF-α when stimulated with trophoblast antigens in vitro (60). In contrast, the supernatant of PBMCs from reproductively normal women contained IL-10, with undetectable levels of Th1 cytokines. These observations of human pregnancy suggest that IL-10 may be an important factor for a healthy, term gestation.

The observed maternal immune tolerance of the genetically foreign fetus in mammalian pregnancy is a complex phenomenon. Undoubtedly, multiple mechanisms are involved in preventing immune rejection of the feto-placental unit. The expression of HLA-G by invasive cytotrophoblasts is one way by which the human placenta can shield the conceptus from an immune attack. In addition to this evasive mechanism, the localized secretion of immunoregulatory cytokines may be very important in dampening an inflammatory immune response. In light of this evidence, it is tempting to speculate that cytotrophoblast-derived IL-10 plays a key role in protecting the allogeneic human fetus in pregnancy.

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